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Supernumerary marker chromosomes characterized by fluorescence in situ hybridization (FISH)

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Abstract. Until recently marker chromosomes have presented a difficult diagnostic problem for cytogeneticists as well as for clinicians. Introduction of FISH to cytogenetic analysis has enabled identification of their origin giving possibility to outline specific phenotypic effects of defined marker chromosomes. Nine marker chromosomes were analysed with FISH using centromeric probes, chromosome-specific libraries and unique DNA sequences probes for PWS/AS critical region. The origin from acrocentric chromosomes was established in 6 cases. One marker was a product of maternal 11;22 translocation and two others were pericentromeric regions of chromosome 2 and 4. Among 6 markers, derived from acrocentric chromosomes, 2 consisted of pericentromeric part of chromosome 15, one was identified as mar (21) and in 3 other cases the origin could not be differentiated between chromosomes 13 and 21 or 14 and 22. Clinical consequences of marker chromosomes including the risk for chromosomal nondisjunction and trisomy 21 as well as the risk for uniparental disomy (UPD) are discussed.

Key words: diagnostic problems, fluorescence in situ hybridization (FISH), marker chromosomes, phenotype-genotype correlation.

Introduction

Constitutional, small supernumerary (accessory) chromosomes of unknown origin are referred to as marker chromosomes (mar). They are detected with a frequency of 0.24/1000 newborns and in 0.4-1.5/1000 of prenatal studies (BUCKTON et al. 1985, SACHS et al. 1987, WARBURTON 1991). Their prevalence

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rate in other defined populations, generally among phenotypically abnormal individuals, is much higher – approximately 2-3/1000 (BUCKTON et al. 1985).

Until recently marker chromosomes have presented a difficult diagnostic problem for cytogeneticists as well as for clinicians. Various attempts were made to classify markers using their cytogenetic characteristics in order to predict possible phenotypic consequences. They were evaluated according to their morphology and staining properties. The mode of origin, the presence of euchromatin together with methods of ascertainment were also taken into account (STEINBACH et al. 1983, BUCKTON et al. 1985, CHEUNG et al. 1990). Nevertheless, none of these methods have proved to be sufficiently reliable to be used in genetic counselling. It is often obscure whether the association of marker chromosome with clinical symptoms such as mild dysmorphic features, mental retardation, recurrent fetal wastage or sterility is causal or coincidental. Thus, the risk for phenotypical anomalies in marker chromosome carriers remains still unclear, especially when de novo mar is discovered at prenatal diagnosis. The risk estimation is currently based on large surveys giving overall and nonspecific risk figures (WARBURTON 1991). Hence, there has been a call for methods outlining specific phenotypic effects of marker chromosomes. The ability to identify their origin, structure and genetic content is a prerequisite to achieve that. In recent years a huge progress has been made by the introduction of fluorescence in situ hybridization (FISH) into cytogenetic analysis. Many marker chromosomes have been characterized with the use of chromosome-specific probes (CROLLA et al. 1992, RAUCH et al. 1992, THARAPEL et al. 1992, BLENNOW et al. 1993, LEANA-COX et al. 1994, CROLLA et. al. 1995). Nevertheless, because of a variety of markers differing in morphology, the identity and possible significance, a large group of them still needs to be fully characterized. Substantially more observations are also needed to confirm previous results to delineate phenotypic effects of marker chromosomes.

We have studied 9 supernumerary marker chromosomes, both familial and de novo. Application of FISH technique enabled us to determine their chromosomal origin.

Material and methods

Nine marker chromosomes detected in the routine cytogenetic studies were reanalysed by in situ hybridization (FISH) technique. A conventional chromosome analysis was performed using standard PHA-stimulated lymphocyte culture followed by a variety of staining techniques (GTG, CBG, DA/DAPI and Ag-NOR).

FISH was performed on conventional cytogenetic 3 to 14-day-old preparations. Standard method of PINKEL et al. (1988) described in detail elsewhere, was used (BOCIAN et al. 1996). Three types of probes were applied in the studies: specific for repetitive DNA sequences (α, β, and classical) from Oncor (Gaithersburgh), chromosome specific libraries from Cambio (Cambridge) and unique DNA sequences for Prader/Willi (PWS)/Angelman (AS) syndrome regions (Oncor). They are listed in Table 1.

Table 1. Probes used

Case	Probe	Region	Locus		Chromosome-specific library	
1	Oncor	cen	D13Z1/D21Z1	+	21	+
2	"	cen	D4Z1	+		–
3	"	cen	D13Z1/D21Z1	+	13,21	?
4	"	cen	D13Z1/D21Z1	+	13,21	?
5	"	cen	D14Z1/D22Z1	+	14,22	?
6	"	cen	D2Z1	+		–
7	"	cen	D14Z1/D22Z1	+	11,22	+,+
8	"	cen	D15Z	+, +/+	15	+
		p	D15F39S6,S7	–		
		p	D15Z1	–		
		PWS/AS	D15S11	+, +/+		
			GABRB3	+, +/+		
9	"	cen	D15Z	+	15	+
		p	D15F39S6,S7	–		
		p	D15Z1	–		
		PWS/AS	D15S11	–		

(+) – positive, (+/+) – positive, two separate signals, ? – inconclusive results.

The probes were labelled with biotin, digoxigenin (DIG) or directly with fluoresceine isothiocyanate (FITC). Hybridization signals were detected using either an avidin-FITC (for biotin) or an anti digoxigenin-FITC (for DIG) fluorescence system. A chromosome analysis was carried out using Nikon epi-fluorescence microscope. Photographs were taken using Kodachrome 400 D or Kodak Ektar 400 and 1000 films.

Results

Conventional characteristics of marker chromosomes as well as their chromosomal origin established using FISH are given in Table 2. Seven out

Table 2. Cytogenetic characteristics of marker chromosomes

Case	Conventional banding analysis						FISH analysis
	Morphology (shape)	Size	CBG	Ag-NOR	DA/DA PI	Chromosomal origin	Karyotype
1	metac	≈22	+	+/+	–	21	47,XX,+mar(21)
2	v.small	<22	+	–	–	4	46,XY/47,XY,+mar(4)
3	metac	≤22	+	+/+	–	13/21	47,XX,+mar(13p/21p)
4	metac	≥22	+	+/+	–	13/21	47,XX,+i(13p/21p)mat
5	metac	<22	+	+/+	–	14/22	47,XX,+mar(14p/22p)
6	v.small	<22	+	–	–	2	47,XX,+mar(2)
7	acroc	<22	+	–	–	11,22	47,XY,+der(22)t(11;22)(q23.3;q11.2)mat
8	variable	<22	+,+/+	–	–	15	46,XY/47,XY+r(15)(p11.1q13)/47,XY, +dic r(15)(p11.1q13)
9	v.small	<22	+	–	–	15	47,XX,t(15;20)(q15;q11.2)+mar(15)

(+) – positive, (+/+) – positive, two separate bands, (–) – negative

of 9 markers originated from acrocentic chromosomes including one being the product of maternal 11;22 translocation. Two others were centromeric regions of chromosomes 2 and 4. The mode of ascertainment, parental origin and description of karyotype are presented below:

Cases 1 and 2: **47,XX+mar(21), 46,XY/47,XY+mar(4)**

47,XX,+mar.ish der (21)(wcp 21+,D13Z1/D21Z1+)
47,XY,+mar/46,XY.ish der (4)(D4Z1+) [ISCN 1995]

Parents of two children with Down syndrome born from the first and second pregnancy. Whether their markers are familial or de novo is unknown. Neither of the trisomic children inherited parental markers. However, the mother's marker was found in one of the two healthy sons. Mother's marker was bisatellited, equal in size to chromosome 22 and chromosome painting documented its origin from chromosome 21 (Fig. 1a, b). Paternal marker was a microchromosome present in 88% of blood lymphocytes. FISH with centromeric probes for several chromosomes showed that microchromosome was in fact a centromeric region of chromosome 4 (Fig. 1 c). In some cells it seemed to be a dicentric ring with two hybridization signals very close to each other.

Case 3: **47,XX,+mar(13p/21p)**

47,XX,+mar.ish der(13p/21p)(wcp13?,wcp21?,D13Z1/D21Z1+)
[ISCN 1995]

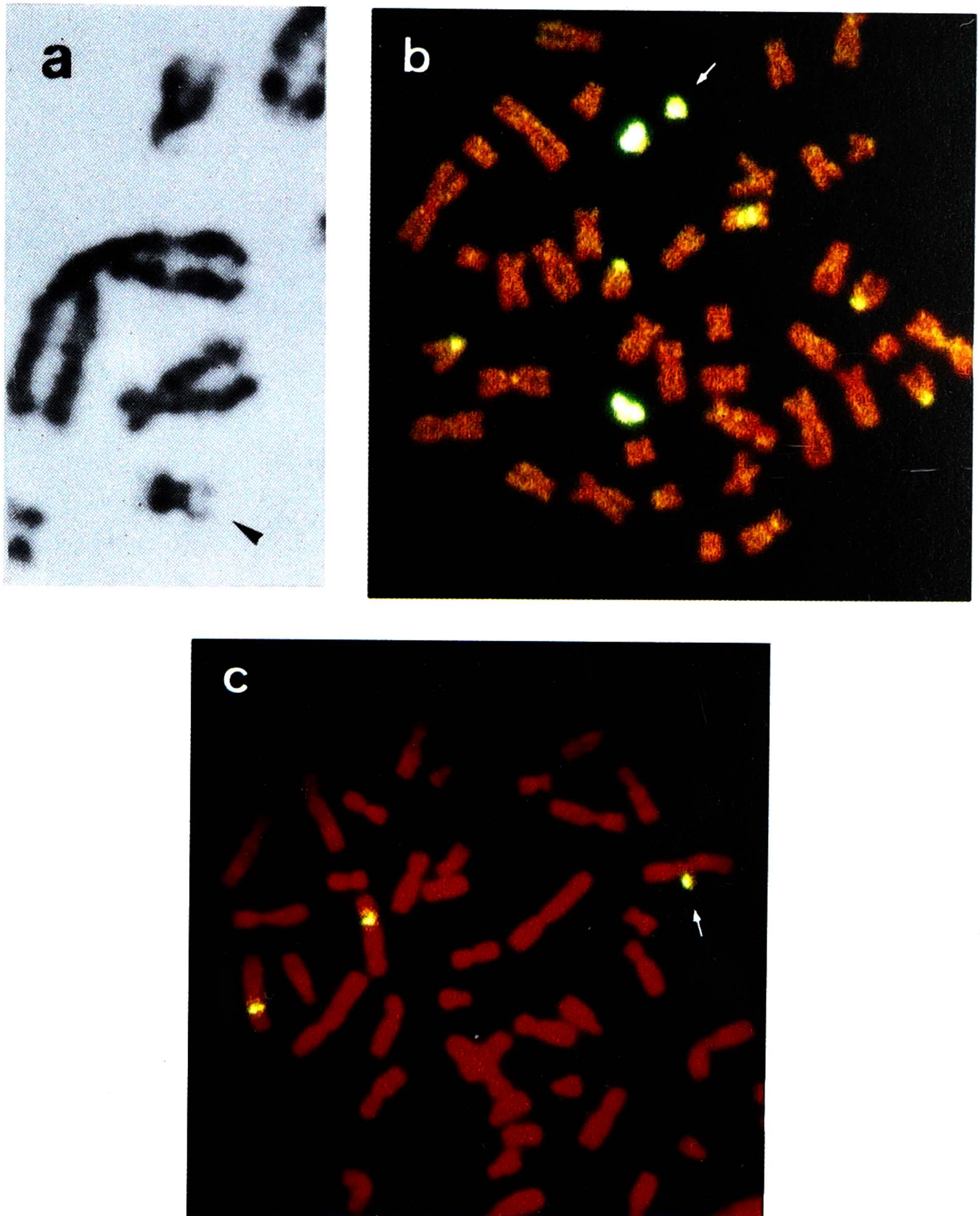
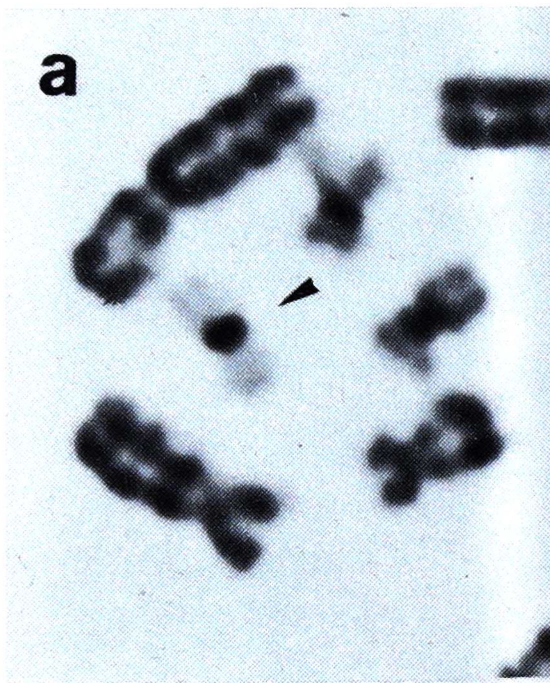
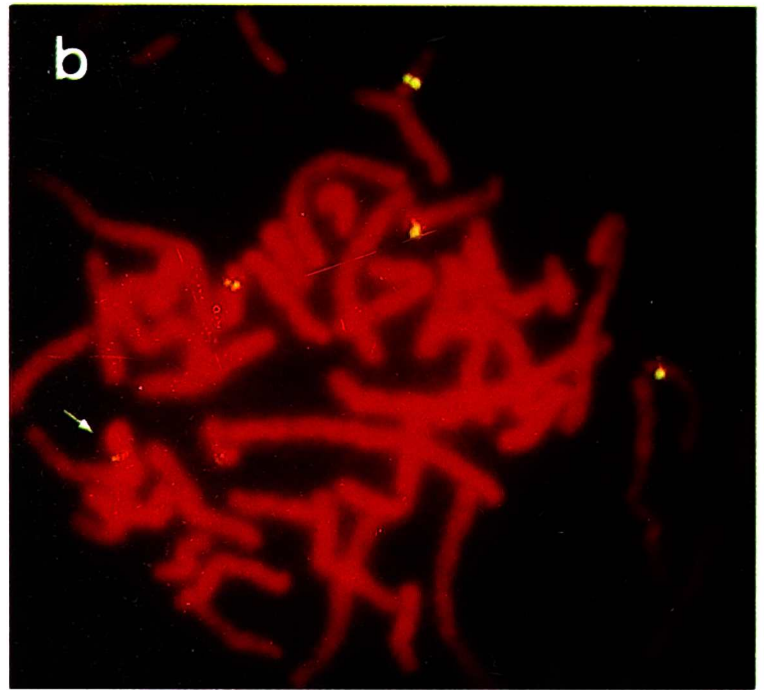


Fig. 1. Marker chromosomes from case 1 (a, b) identified as mar(21) and from case 2 (c) delineated as mar(4) using FISH

Markers are indicated with arrows: (a) – GTG banding, b – chromosome 21 specific library painting two homologues and the marker, c – D4Z1 pericentric repeat probe showing hybridization signal at centromeres of two homologues and on the marker





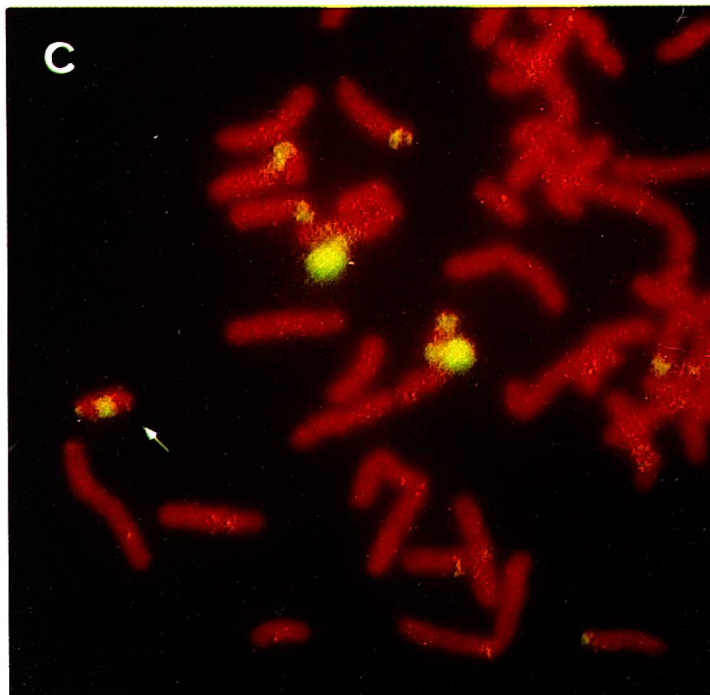
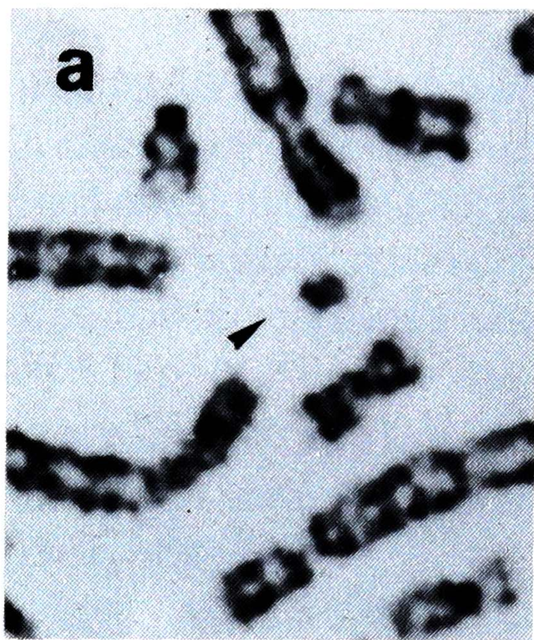
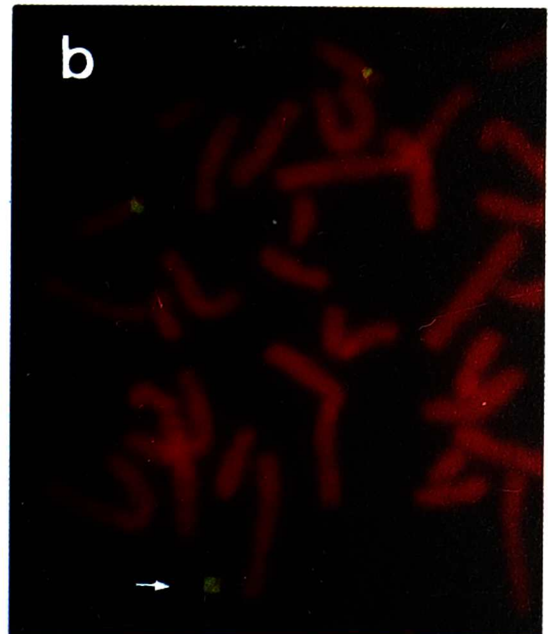
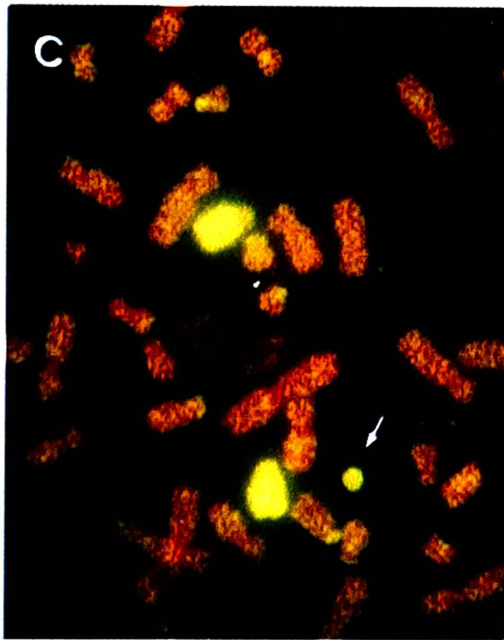
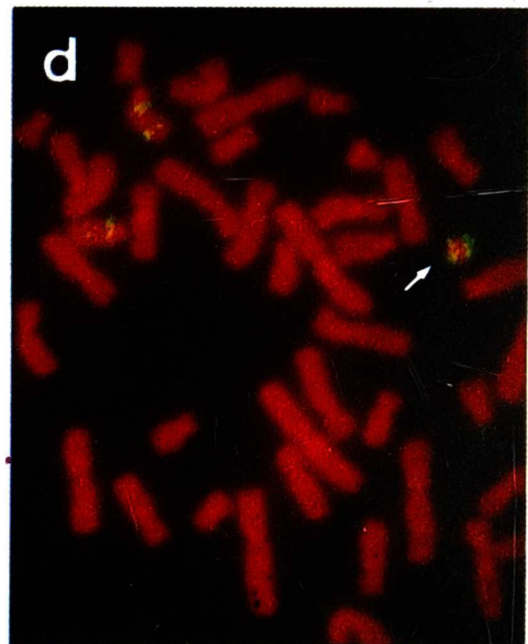


Fig. 2. Marker chromosome from case 4 identified as i(13p/21p) using FISH
Marker is indicated with an arrow: (a) – GTG banding, (b) – D13Z1/D21Z1 centromeric repeat probe showing hybridization signal at centromeres of two homologues 13, 21 and the marker (c) – chromosome 21 specific library painting strongly two homologues and weaker marker as well as pericentromeric regions of other acrocentrics









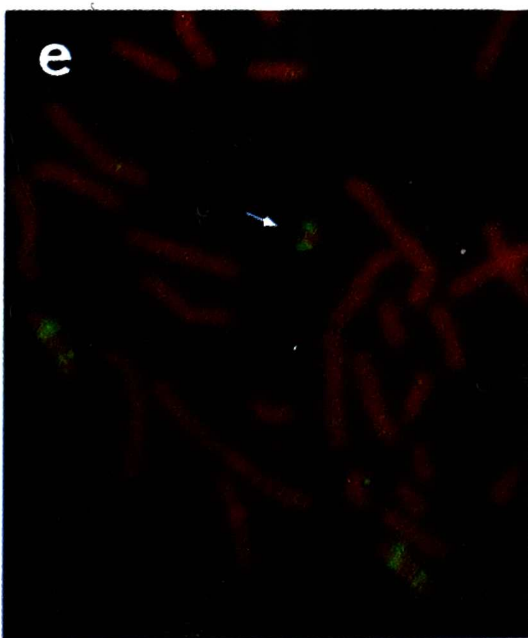


Fig. 3. Marker chromosome from case 8 identified as dic r(15)(p11.1q13) using FISH

The marker is indicated with an arrow: (a) – GTG banding, (b) – D15Z centromeric repeat probe showing hybridization signal on two homologues and on the marker (c) – chromosome 15 specific library painting two homologues and the whole marker (d,e) D15S11 and GABRB3 PWS/AS region specific cosmids with PML chromosome 15 control probe. The proximal signal on two homologues shows the presence of PWS/AS region and the distal one identifies chromosome 15. The marker shows two hybridization signals specific for PWS/AS regions documenting duplication of this region.

Mother of the proband with Down syndrome due to a 21/21 translocation. The origin of the marker is not known. The family history showed spontaneous abortions and one stillbirth in the grandmother of the proband. Her karyotype was normal. The grandfather was not available for the study. Marker was half the size of chromosome 22 and bisatellited. FISH documented its origin from chromosome 13 or 21.

Case 4: **48,XX,+21,+i(13p/21p)mat**
48,XX,+21,+mar.ish i(13p/21p)(wcp13?,wcp21?,D13Z1/D21Z1+) [ISCN1995]

The proband was a girl with Down syndrome. Besides trisomy 21 she carried a marker chromosome which was maternal in the origin. The same marker was also found by proband's healthy brother. The marker was symmetrical, bisatellited and had the size of F group chromosomes. Its morphology and the results of FISH showed it was an isochromosome of the short arms of chromosome 13 or 21 (Fig. 2).

Case 5: **47,XX,+mar(14/22)**
47,XX,+mar.ish der(14p/22p)(wcp14?,wcp22?,D14Z1/D22Z1+) [ISCN 1995]

The marker was ascertained because of 5 spontaneous abortions in a 32-year-old female. The same marker chromosome was found in her healthy son. The origin of the marker is unknown. It was smaller than chromosome 22 and bisatellited. FISH showed that marker originated from chromosome 14 or 22.

Case 6: **47,XX,+mar(2)**
47,XX,+mar.ish der(2)(D2Z1+) [ISCN 1995]

The patient was referred at the age of 36 years because of fetal wastage. A very small marker (p arm of acrocentrics in size) was found in peripheral blood lymphocytes. The origin of the marker was not studied. A systematic search with centromeric probes for several chromosomes showed that the marker was a centromeric region of chromosome 2 probably with a ring structure and two centromeres in some cells.

Case 7: **47,XY,+der(22)t(11;22)(q23.3;q11.2)mat**
47,XY,+mar.ish der(22)t(11;22)(q23.3;q11.2)mat (wcp22+,D14Z1/D22Z1+,wcp 11+) [ISCN 1995]

The proband was referred at the age of two months for hypotonia and dysmorphic features. At present at the age of 2.5 years he shows a marked developmental delay. The case was presented elsewhere (STANKIEWICZ et al. 1996).

Case 8: **46,XY/47,XY,+r(15)(p11.1q13)/47,XY,+dic r(15)(p11.1q13) de novo**
47,XY,+ mar/46,XY.ish r(15)(wcp 15+,D15F39S6-,D15Z1-,D15Z+, D15S11+,GABRB3+[40]/r(15)(wcp 15+,D15F39S6-,D15Z1-,D15Z++, D15S11++,GABRB3++[30]/46,XY[30] [ISCN 1995]

The proband was referred at the age of 13 years because of mental retardation, mild dysmorphic features and short stature. Mosaic karyotype showed the presence of a very small marker chromosome with variable morphology in 70% of lymphocytes. As it was NOR and DAPI negative, systematic studies with centromeric probes for all but acrocentric chromosomes were performed. Because of negative results of the studies, probes for acrocentrics were also used. The marker chromosome appeared to be a monocentric and in some cells – a dicentric fragment (ring) of chromosome 15. The application of different probes for that chromosome (Table 1) allowed to localise breakpoints at p11.1 and q13 (Fig. 3).

Case 9: **47,XX,t(15;20)(q15;p11.2),+r(15)(p11.1q11.2-12)pat**
47,XX,t(15;20)(q15;p11.2),+mar pat.ish der(15)(wcp 15+,D15F39S6-, D15Z1-,D15Z+,D15S11-) [ISCN 1995]

The proband was referred at the age of one year and eight months because of hypotonia, mild dysmorphic features and psychomotor retardation. Balanced reciprocal translocation and a very small marker chromosome were found. Both chromosome abnormalities were inherited from the healthy father. The marker chromosome was also found in the proband's brother with Klinefelter syndrome who was also a paternal translocation carrier. FISH with various probes (Table 1) documented that the marker consisted of a centromeric region of chromosome 15.

Discussion

The origin structure and genetic content of marker chromosomes

Large population studies with conventional methods have shown that 86% of markers are derived from acrocentric chromosomes, approximately half of which include the short arm region of chromosome 15 (BUCKTON et al. 1985). In our group, 7 of 9 markers originated from acrocentric chromosomes and two of them consisted of a pericentromeric part of chromosome 15. Four markers were bisatellited, whereas three were NOR negative. Two of these NOR as well as DAPI negative markers appeared to be derived from chromosome 15. It is not unexpected because in both mar(15) chromosomes the deletion of a short arm was shown with β and satellite III DNA probes. In the third

NOR negative case marker was a product of 11;22 translocation consisting of 22pter → q11.2 and 11q23.3 → qter. Likewise reports of other authors, our observations, show that the lack of NORs not always means that marker originates from other than acrocentric chromosomes (BLENNOW et al. 1995). Similarly, markers derived from chromosome 15 may be negative for both, DAPI and Ag-NOR staining. Thus, when the origin of a marker chromosome is studied, even in the cases without satellites, it seems reasonable to start investigations with centromeric probes specific for acrocentric chromosomes.

Unfortunately, commercially available centromeric probes for acrocentric chromosomes were not specific enough to differentiate between 13 and 21 or 14 and 22 chromosomes. Out of four markers (case 1, 3, 4, 5) derived from these chromosomes only one (case 1) could be specified by painting with chromosome 21 library. In cases 3 and 4, hybridization signal from 21 specific library seemed to be stronger than that from library specific for chromosome 13 and from signal caused by cross hybridization to the short arms of other acrocentric chromosomes. However, the difference was not convincing enough to identify markers as derived from chromosome 21. In case 5 discussed above the results of painting with library for chromosomes 14 and 22 did not indicate which chromosome the marker is derived from. Cytogenetic characteristics and results of FISH point, that all these markers are composed of centromeres and short arms of acrocentrics. In case 4, the marker was symmetrical and delineated as isochromosome 13 or 21 p. More specific probes and experimental conditions are necessary to define precisely markers consisting mainly of heterochromatin from pericentromeric regions of chromosomes 13, 14, 21 and 22 (CALLEN et al. 1992).

Chromosome specific libraries should be used along with centromeric probes because they may give additional information on the origin of a marker chromosome (as in case 1). Finally, especially in the case of large markers, more than one chromosome may be involved in the rearrangement (our case 7, BLENNOW et al. 1992) which is also a reason for painting chromosomes along with the use of centromeric probes.

The composition of two markers derived from chromosome 15 (case 8 and 9) could be established using various probes specific for the p-arm and q-arm (for PWS/AS region). In case 8, the marker was unstable and showed a mono- or dicentric ring structure consisting of centromere(s) and PWS/AS region 15q11-13. However, in case 9 the marker appeared to be composed almost entirely of alphoid centromeric heterochromatin. It was negative when tested with D15Z1 as well as specific for PWS region D15S11 cosmid probe.

It is known that chromosome 15 is most often involved in the formation of marker chromosomes. Over 50% of all markers are identified as inverted

duplications (15)(pter→q11-13) (BUCKTON et al. 1985). Our two mar(15) chromosomes and those described by CROLLA et al. (1995) proved that there is considerable variability in the size, structure and molecular composition of markers derived from chromosome 15. These markers were divided into six sub-groups (CROLLA et al.1995). Case 8 could be of II category in some cells although due to a small size of the marker it is difficult to define its structure. It seems reasonable to assume a ring structure in both mono- and dicentric forms as the deletion of p and q-arm create sticky ends which are suggested to join. At least in 30% of lymphocytes the marker chromosome was dicentric and in situ hybridization with D15S11 and GABRB3 probes showed duplication of this region (Fig. 3 d, e). If it is tandem or inverted duplication it could be elucidated using simultaneously two colour FISH with centromeric and PWS/AS critical region specific probes. Distal position of two hybridization signals seen in many cells when probes for PWS/AS critical region were used, indicates rather inverted than tandem duplication. To the best of our knowledge it is the first case with such a marker derived from chromosome 15 and it does not fit to any of the six categories of CROLLA et al. (1995). Our second mar(15)(case 9) is of VI category in their classification.

Phenotype – genotype correlations

Until recently phenotypic consequences of a supernumerary marker chromosomes have been difficult for evaluation. Knowledge of chromosomal origin of markers is the first step towards elucidation of phenotype-genotype correlation. Phenotypes of six (cases 1-6) out of nine identified in these studies marker chromosome carriers were normal. In general, clinical symptoms associated with marker chromosome depend on the genetic content of the marker in each separate case. If the marker consists entirely of heterochromatin it is expected to have no effect on the phenotype. This is the case of our four markers derived from the short arms of chromosomes 13/21, 14/22 as well as two markers composed probably of purely centromeric heterochromatin of chromosomes 4 and 2 (cases 2 and 6, respectively). Other authors have also shown that satellited markers derived from chromosomes 13 or 21 and 14 appeared to have a low risk, however, such markers originated from chromosome 22 are associated with a high risk of phenotypic abnormalities (CALLEN et al. 1992, BLENNOW et al. 1995).

In one (case 9) out of three our cases with abnormal phenotype (cases 7, 8 and 9) the marker does not seem to be causally associated with the observed abnormality. The marker was mostly composed of centromeric heterochromatin

of chromosome 15 and inherited from a phenotypically normal father. Such small mar(15) was observed to be harmless (LEANA-COX et al. 1994, BLENNOW et al. 1995, CROLLA et al. 1995) in other studies. Clinical manifestations in case 8 with mar(15) confirm observations on association between the presence of proximal 15q region (three or four copy numbers of this region) and abnormal phenotype (BLENNOW et al. 1994, LEANA-COX et al. 1994, BLENNOW et al. 1995). Mental retardation, hyperactivity, aggressiveness, deficits in language development, down slanting palpebral fissures and short stature observed in our patient were also present in other cases with markers identified as inverted duplication of (15)(pter → q12-13) (BLENNOW et al. 1995). Clinical features of the carrier of der(22)t(11;22) marker (case 7) were consistent with symptoms characteristic of partial trisomy of chromosomes 22 and 11 and were discussed elsewhere (STANKIEWICZ et al. 1996).

Large numbers of cases with identified markers which are correlated with a clinical picture of the patients have to be described to provide risk estimates reliable enough to be used in genetic counselling. In determining a potential clinical risk associated with markers, limitations due to a possible ascertainment bias have to be also recognized.

One of the questions to be answered in evaluating a potential risk for marker chromosome carriers is whether markers may interfere with normal chromosome disjunction at meiosis resulting in aneuploidy as it was suggested earlier (RAMOS et al. 1979, ANNEREN et al. 1984, BUCKTON et al. 1985). There have been several reports of families with Down syndrome in most of which mothers were bisatellited marker chromosome carriers (STEINBACH et al. 1983, ANNEREN et. al. 1984, BUCKTON et al. 1985, HOWARD, FIELDING 1987, SACHS et al. 1987). In one third of these families trisomy 21 appeared more than once what might suggest a greater risk of nondisjunction. On the other side, such observations could be the result of a severe ascertainment bias (STEINBACH, DJALALI 1983).

It has also been suggested that the marker chromosomes in families with Down syndrome might be isochromosomes of the short arm of chromosome 21 (ANNEREN et al. 1984). These studies were conducted on three families with Down syndrome in which mothers were bisatellited marker chromosome carriers. In one of them the marker was proved to derive from chromosome 21 (case 1). However in two others, although we thought they could be mar(21) as it was discussed earlier, the marker origin was not clear and the markers were suggested to derive from chromosome 21 or 13. Theoretically in the both cases, the influence of these markers on meiotic segregation of chromosome 21 could be explained.

A great homology between repetitive DNA sequences of centromeres and p arms of chromosomes 13 and 21 may allow pairing and exchange of genetic material between these two chromosomes at meiosis (CHOO 1990). Thus, markers derived from 13p or 21p could pair with the short arms of two normal acrocentric homologues and form a complex three-chromosome configuration (SPEED 1984, CROLLA et al. 1995). Such anomalous pairing would disturb normal segregation and lead to non-disjunction. If this model is correct, carriers of marker chromosomes may produce disomic or otherwise unbalanced gametes leading to fetal wastage as suggested by BUCKTON et al. (1985). The proportion of disomic gametes will produce, by the mechanism of "rescue" from the trisomic state, a uniparental disomic (UPD) embryo which may be associated with abnormal phenotype as in PWS/AS syndromes (ROBINSON et al. 1993, JAMES et al. 1995). Whether supernumerary marker chromosomes predispose to an increased risk of a concomitant UPD for normal homologues which may be associated with growth defects needs elucidation.

In conclusion, results of these studies have demonstrated the utility of molecular cytogenetic investigations in the identification of marker chromosome origin. When more well-mapped single copy probes are available, a more detailed analysis of marker composition would be possible. FISH analysis of more cases with markers should lead to a more accurate assessment of the association between the presence of a marker of defined origin and abnormal phenotype. Other possible consequences of markers such as nondisjunction resulting in aneuploidy which may be associated with an increased risk of UPD and dosage effect have to be elucidated before an accurate risk estimate for any given de novo marker is given.

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